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14. ABSTRACT This is our annual report for the project: P11, A Biomarker for Memory Retrieval: A Possible Role in Traumatic Stress. We carried out all of the experiments based on the proposed research design. Due to all experiment dependent on the p11 knockout mice and the postponed scheduled p11 knockout mice development, we have tested memory retrieval performance with wild type mice. Fortunately, the detailed plan of p11 knockout mice development, such genotyping and delivery schedule has been approved by both our institution and The Jackson Laboratory (JAX). Currently, the p11 knockout mice have been breeding now at (JAX). As we proposed, we will use the p11 knockout mice to conduct two critical experiments to determine the effects of footshock on water-maze spatial task performance and to examine the role of p11 in memory retrieval performance in these mice. Our data from wild type of mice is the same as pervious reported: (1) Footshock and corticosterone significantly decreased the time spent on target and produced no effect on time spent on opposite, indicating a significant impaired performance in the water-maze spatial task in stressed mice compared to control in p11 wild type. (2) Stress resulted in p11 protein up-regulation, which as determined by Western Blot in the hippocampus, cortex and amygdala of wild type mice. (3) Stress and corticosterone resulted in p11 mRNA up-regulation, which as determined by real-time PCR in the hippocampus, a brain region associated with memory function. The ongoing final experiment with p11 knockout mice will allow us to accomplish all of our proposed specific aims.					
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INTRODUCTION

Subject: Pre-clinical and clinical studies have shown the important role of p11 in Post-Traumatic Stress Disorder (PTSD) (Zhang 2008, 2009. CSTS, News From the Center, 2008; CSTS, 2008 Annual Report; Army Annual Report 2009 and Psychiatric News Oct 2, 2009). P11, also known as S100A10, is a protein encoded by the S100A10 gene in humans and is a member of the S100 family of proteins containing two EF-hand calcium-binding motifs, which are localized in the cytoplasm and/or nucleus in a wide range of cells (Santamaria-Kisiel et al., 2006). P11 is associated with both depression (Svenningsson et al., 2006) and PTSD (Zhang et al., 2008; Su et al., 2009). The evidence from animal studies supports the view that stress influences learning and memory (de Quervain et al., 1998) and increases p11 expression in the brain (Zhang et al., 2008). The most common characteristic of PTSD is the reexperiencing syndrome, wherein the patient's memory seems to be fixed on a traumatic event such that the processing of non-trauma-related memories is often impaired. Therefore, these results led us to hypothesize that p11 might play an important role in memory. It is found that footshock given 30 minutes before memory testing impaired rats' retention performance in a water-maze spatial task, compared to non-stressed controls. This impaired retention performance corresponded to the levels of circulating corticosterone at the time of retention testing. In addition, the stress-induced retention impairment was blocked by metyrapone, a synthetic inhibitor of corticosterone. Furthermore, systemic corticosterone administered to non-stressed rats 30 min before retention testing induced dose-dependent retention impairment (de Quervain et al., 1998). Finally, glucocorticoid increases p11 expression *in vitro* through glucocorticoid response element sites in the p11 gene promoter (Zhang et al., 2008). Therefore, we will use wild type and p11 knock-out mice in this study to elucidate the possible role of p11, which can be regulated by glucocorticoid, in memory retrieval with the objective of facilitating the development of a therapeutic intervention for stress related disorder PTSD.

Purpose: Our immediate objective is to use the p11 knockout stressed animal model, which was developed in our laboratory, in collaboration with the Jackson Laboratory, to investigate the role of p11 in memory. Our study will provide an opportunity to determine the possible mechanism of stress-induced changes of retrieval memory, which are mediated by p11 and glucocorticoids. This information may translate into knowledge that will help to develop medicines for PTSD treatment. Military personnel who are exposed to trauma at higher-than-average frequencies need to have an efficient medicine to help minimize PTSD. The evaluation for the effect of p11 regulated by glucocorticoid on memory may provide an alternative and/or adjunctive therapy for PTSD seen in military psychiatry.

Scope:

- **Innovation:** This study will provide information about the molecular mechanism underlying memory in stress and fill a knowledge gap in the current research on PTSD. Such knowledge may facilitate the development of novel pharmacological interventions for the treatment of PTSD.
- **Intervention:** Administration of glucocorticoid agents targeting p11 gene may provide treatment of fear memory in PTSD while minimizing side effects.
- **Application:** Interventions of p11 gene expression have the potential for use both in military populations (those on active duty, reservists and veterans) and in civilian populations exposed to traumatic stress (natural disasters, vehicle crashes, etc.).

BODY

Task 1: Animal protocol approvals

Announcement of Concept Award W81XWH-08-2-0202 to Lei Zhang 2008. The Certificate of Environmental Compliance for this project was awarded by the USUHS Environmental Compliance Officer in 2008 and the PI Assurance Document was signed April 29, 2008. Our animal protocol was approved on September 29, 2008. A copy of the IACUC approved animal protocol was submitted to the USAMRMC ACURO for review and got ACURO approval. Since this experiment is using p11 knockout mice and the mouse is not available in the market, we have to develop a p11 knockout animal protocol of our own. Development of a p11 knockout will provide a great opportunity to study the possible role of p11 in memory and in PTSD. However it is also a challenge, scientifically and administratively. Originally, we planned to develop p11 knock-out mice at Childrens' National Medical Center in Washington, DC, considering their low cost, proximity, and expertise.

Unfortunately, there was a change of personnel responsible for breeding our p11 knock-out mice in the hospital, so we had to search for a new knock-out facility. We have searched several possible companies, but the cost for development p11 knock-out mice is much higher than we had proposed. Fortunately, through long negotiations and discussions of the technical issue on the development of p11 knock-out mice, we found that the Jackson Laboratory can develop p11 knockout mice within our budget. The Jackson Laboratory is an independent, nonprofit organization focusing on mammalian genetics research to advance human health. Their mission is to discover the genetic basis for preventing, treating and curing human disease, and to enable research for the global biomedical community. They have more than 1,400 employees located in Bar Harbor, Maine, and in Sacramento, California and conduct genetic research, providing scientific services and genetic resources to laboratories around the world. They have used wild type and knock-out mice as research tools. The mouse genome is 95 percent identical to the human genome and is therefore an effective and efficient model for human diseases. Jackson Laboratory is the major provider of knock-out mice in the US. Since development of a p11 knockout mouse is an innovation, there is intellectual (material transfer) property issue. A material transfer agreement is required from all participating institutions. We are not able to order the p11 knockout mice from the Jackson Lab until the agreement is signed by the institutions. We, and our university's Joint Office of Technology Transfer have been negotiating with Jackson Lab since August 9, 2010. We proposed to order specific frozen mice embryos from MRC Harwell to create a p11 knockout mice at The Jackson Lab. The p11 knockout mice will be bred and genotyped at Jackson Lab. The final agreement was reached by all sides on March 9, 2011. The p11 knockout mice will be bred and genotyped at Jackson Lab. Meanwhile, we have conducted experiments using wild type mice. Those experiments are part of our proposal. All animal experiments have been done in accordance with IACUC and ACURO approved protocols. Since last report, there is a program for p11 knockout mice. The

Task 2. To determine the effects of pretest administration of footshock stress or systemic corticosterone (the major glucocorticoid) on memory retrieval performance. We have examined the effects of pretest administration of footshock stress and systemic corticosterone (the major glucocorticoid) on memory retrieval performance and p11 expression in the mouse brain in p11 wild type mice, as we proposed. In this experiment, after footshock or treatment with corticosterone, memory performance was examined using a spatial water-maze procedure, then p11 expression levels in the hippocampus, amygdala and cortex of the mouse were determined by Western blot and real-time PCR. In this experiment, after a footshock or treatment with corticosterone, memory performance was examined using a spatial water-maze procedure. Then, p11 expression levels in the hippocampus, amygdala and cortex of the mouse were determined by Western blot and real-time PCR. All mice were housed in group cages (2-3) with free access to food and water. They were kept in the air-conditioned animal facility with a 12 hr light/dark cycle. All mice were identified by cage card. Body weight was taken before and after experiments.

It is expected that footshock or corticosterone will impair memory retrieval and alter the expression of p11 in discrete regions of the mouse brain. If stress-induced memory retrieval impairment does not alter p11 expression, such findings would indicate that stress-induced retrieval impairment is independent of glucocorticoid-regulated P11 expression. The results will be confirmed in the proposed study with p11 knockout mice, when they are available in our lab. The procedures using animals have been approved by IACUC of our university. Briefly, a mouse was placed in a dark shock chamber and is acclimatized for 5 Min. The mouse was then subjected to 120 shock trials from which it cannot escape. Each shock (the unconditioned stimulus) is 0.45mA, lasts 15 seconds and is preceded by 3 seconds of a cue light (the conditioned stimulus). The period of time between shocks are randomized, between 22 and 68 seconds, and average 45 seconds.

In the memory test, the animals were placed into the water at and facing the sidewalls of the pool, at different start positions across trials, where they quickly learn to swim to the correct location with decreasing escape latencies by more direct swim paths. The tracking system measures the gradually declining escape latency across trials, and parameters such as path-length, swim-speed, and directionality in relation to platform location. Mice received one training session each day for 10 d. Each daily session began with a single reinforced probe trial, followed by four training trials. For the probe trials, the platform was lowered so that it was inaccessible and the mouse was placed in the water facing the pool wall at one of four start points (north, south, east, or west). The start points were counterbalanced across trials for all animals. Upon release into the

water, the mouse was allowed to swim for 60 sec, at which point the platform was raised to within 1.5 cm of the water surface. An additional 60 sec were then allowed for the mouse to locate the platform and escape from the water. After escaping, the mouse remained on the platform for 30 sec before being removed. If the mouse fails to escape, it was guided to the platform and remained there for 30 sec. After completion of the daily probe trial, four training trials were given with the platform in the raised position (1.5 cm below the water surface) so that it will provide a means of escape from the water. The procedure was the same as for the probe trials, except that the mouse was allowed 120 sec to find the platform. On completion of training, mice were assigned to an immediate stress or non-stressed group. Stressed mice were stressed with food shock. Non-stressed mice were used as controls. We conducted a probe trial in which the escape platform was removed from the pool and each animal allowed to swim for 60 sec. A well-trained mouse swam to the target quadrant of the pool and repeatedly across the former location of the platform until starting to search elsewhere. This spatial bias will be used to constitute evidence for spatial memory. We found that footshock (30 min) resulted a significant decrease in the time spent on target, while producing no effect on time spent on opposite, indicating that stressed mice had significant impaired performance in the water-maze spatial task compared to control in p11 wild type (Fig 1).

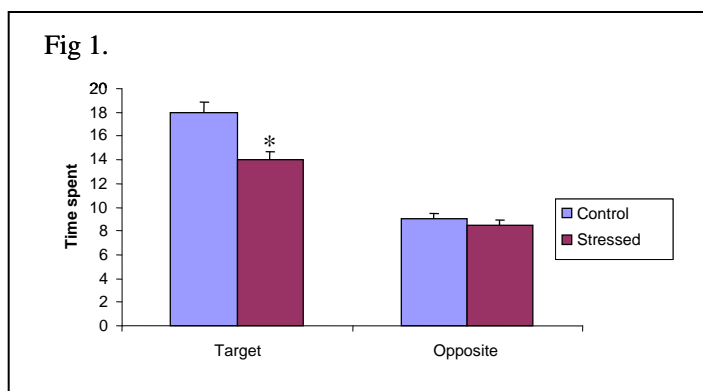


Fig 1. The effect of stress on memory retrieval. Mice had impaired performance in the water-maze spatial task after being given footshock 30 min compared to control in p11 wild type mice. $p < 0.05$

In this experiment, we also carried out the water-maze spatial task performance of animals treated with saline, corticosterone and found corticosterone treatment resulted significant decrease of time spent, indicating a glucocorticoid-induced impaired memory retrieval performance (Fig 2).

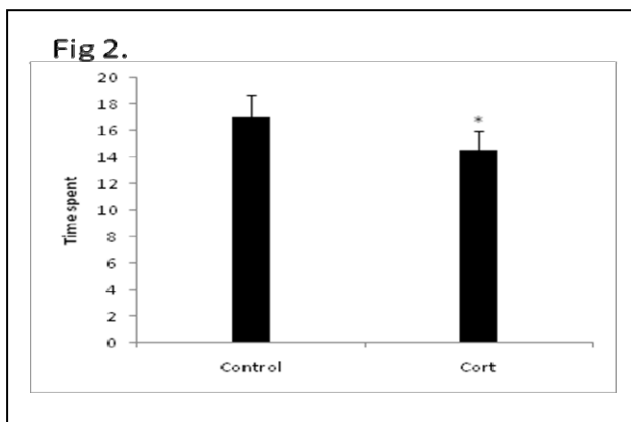


Fig 2. Effect of corticosterone on retention impairment. Cort, corticosterone. $p < 0.05$

Task 3. To determine the effects of pretest administration of footshock stress on p11 expression in the mouse brain by Western Blot. We also examined the effect of stress on p11 expression in the hippocampus and amygdala by Western blot. The procedure for this experiment follows in the proposed methods. Briefly, control (n=10) and stressed mice (n=10) were decapitated. The brain was excised from each and sliced coronally using a vibratome. The hippocampus and amygdala were dissected from the slices and placed on dry ice immediately for Western blot and p11 mRNA expression studies. Protein concentration in the samples was determined by Bio-Rad Protein Concentration Reagent (Hercules, CA). Equal amounts of total protein (20 µg per lane) were resolved in 10% SDS polyacrylamide gels and blotted onto PVDF membranes for immunoblotting analysis. Protein expression was detected using a 1:500 dilution of mouse anti-p11 monoclonal antibody (BD Transduction Laboratories, Franklin Lakes, NJ) with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG as a secondary antibody (Bio-Rad Laboratories, Hercules, CA). The density values are presented as means ± S.D. from three experiments. The density was used to quantify immunoreactivity in terms of percentage of p11 induction relative to control (non-stressed mice).

Using Western blot, we found that stress results in increases of p11 expression in the three brain regions, indicating that p11 was up-regulated by stress exposure in wild type mice (Fig3).

Fig 3.

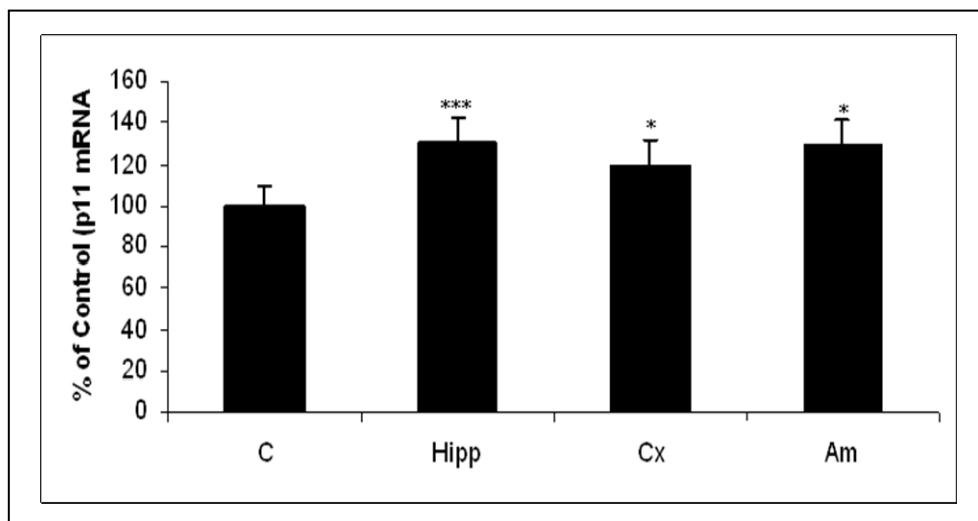


Fig 3. Stress resulted in p11 up-regulation, as determined by Western Blot in the hippocampus and amygdala of wild type mice. The data were analyzed by Student's *t*-test; * $P < 0.05$, *** $P < 0.001$. C, Control; Hipp, Hippocampus; Cx, Cortex, Am, Amygdala.

Task 4. To determine p11 mRNA expression in the mouse brain by real-time PCR. In this experiment, after footshock or treatment with corticosterone, memory performance will be examined using a spatial water-maze procedure, then p11 mRNA expression levels in the hippocampus, amygdala and cortex of the mouse will be determined by real-time PCR. In our lab brain tissue is placed in a 10 ml Wheaton® glass homogenization tube, which increases the entire volume (tissue + buffer) to a total of 2 ml with the homogenization buffer. An appropriate sized pestle is placed in the homogenization tube containing the organ and buffer. Brain tissue is then homogenized using the tissue grinder: RW16 Basic S1 Overhead stirrer (IKA® works Inc., Wilmington NC) set at a speed of approximately 1300 rpm. Going up and down the tube only twice, the pestle keeps each

sample homogenous. Based on the volume, homogenate is poured into a 2 ml tube and sonicated for 30 seconds with the VirSonic sonicator.

We use organic extraction protocols to begin the lysis and homogenization of brain tissues in a proprietary, monophasic solution of phenol and guanidinium isothiocyanate. Chloroform is then added, followed by centrifugation to separate the RNA from gDNA and proteins. The aqueous phase is removed and the RNA is precipitated, washed, and solubilized in RNase-free water. Using organic extraction, this procedure yields RNA of the highest quality.

At the end of the extraction procedure, quantitation is done by taking the spectrophotometric absorbance at 260 nm (Eppendorf Biophotometer). Assessment of RNA quality is done by both electrophoresis and the calculation of a spectrophotometric A260/A280 ratio. The A260/A280 ratio falls in the range of 1.8 - 2.2. Electrophoresis is performed on an agarose gel for samples in which 1ug can be spared for the analysis. Electrophoresis results show two strong, distinct bands representing ribosomal RNA and a light smear behind the ribosomal bands representing messenger RNA. In addition, significantly higher molecular weight products indicative of contaminated genomic DNA are not observed.

RNA was extracted from mouse hippocampus and amygdala tissue lysates using TRIzol. cDNA was generated from 3 ug of total RNA using Superscript III RT (reverse transcriptase) and oligo (dT) primers (Invitrogen). Real-time PCR was performed on the generated cDNA product in the IQ5 system using SYBR Green (Bio-Rad). The following sequences for p11 mRNA analyses were used: forward 5'-TGCTCATGGAAAGGGAGTTC-3' and reverse 5'-CCCCGCCACTAGTGATAGAA-3' primers. Beta-actin mRNA levels were unchanged by treatment and were used as an internal control for normalizing p11 mRNA levels in control and experimental samples. Sequences for beta-actin primers were as described by Applied Biosystems. Dilution curves confirmed the linear dependence of the threshold cycle number on the concentration of template RNAs. Relative quantification of p11 mRNA in control and experimental samples was obtained using the standard curve method. Statistics were performed using GraphPad Prism (GraphPad Software, Inc. San Diego, CA).

We found stress and treatment of corticosterone resulted in increases of p11 mRNA expression levels in the hippocampus of wild type mice. Our results suggested stress up-regulated p11 at gene levels in the hippocampus, a brain region associated with memory (Fig4).

Fig 4.

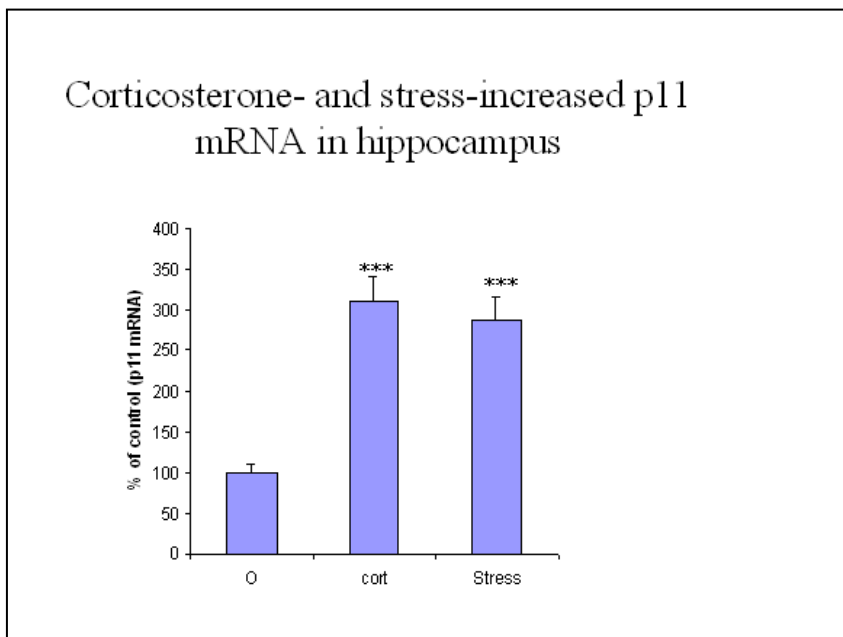


Fig. 4 The effects of stress and corticosterone on the p11 mRNA levels in mice hippocampus. Both stress and corticosterone (Cort) increased p11 expression levels in wild type mice hippocampus. The data were analyzed by Student's *t*-test; $p < 0.05$

Summary of Experimental Results of Task 2, 3 and 4

- Footshock and corticosterone significantly decreased the time spent on target and produced no effect on time spent on opposite, indicating a significant impaired performance in the water-maze spatial task in stressed mice compared to control in p11 wild type.
- Stress resulted in p11 protein up-regulation, as determined by Western Blot in the hippocampus, cortex and amygdala of wild type mice.
- Stress and corticosterone resulted in p11 mRNA up-regulation, as determined by real-time PCR in the hippocampus, a brain region associated with memory function.

KEY RESEARCH ACCOMPLISHMENTS May 1, 2008 through Sept. 14, 2011

- To get Knockout mice and footshock protocol approval at USUHS.
- Design and negotiate with companies to develop our own p11 knockout mice.
- Examine the effect of footshock and corticosterone on memory preference in wild type mice using proposed protocol.
- Examine the effect of footshock on p11 protein expression in the three brain regions which are associated with memory in wild type mice using Western Blot.
- P11 antibodies and Western Blot procedure were tested and validated in wild type mice.
- Examine the effect of footshock and corticosterone on p11 mRNA expression in hippocampus, which plays a major role in memory retrieval.
- Real time PCR procedure were tested and validated in wild type mice.
- Breeding and genotyping of P11 knockout mice is begun

REPORTABLE OUTCOMES

- Oral and poster presentation at the Military Health Research Forum, August 31-September 3, 2009 in Kansas City, MO.

CONCLUSION

Our results indicate that besides the well described effects of stress and glucocorticoids on the acquisition and consolidation processes, stress and glucocorticoids also affect memory retrieval mechanisms, p11 protein expression in the hippocampus, cortex and amygdala and p11 mRNA expression in the hippocampus in wild type mice. Stress and glucocorticoids resulted in p11 over expression in the mouse brain. The detailed molecular mechanism of p11 in memory retrieval will be determined in p11 knock-out mice, which is our on-going task. We expect that the p11 knock-out mice will be available and the comparison of memory retrieval between wild type and p11 knock-out mice will help us to understand the possible role of p11 in memory. This will help in the development of a strategy for the treatment of stress related disorders such as depression and PTSD.

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